EXHIBIT 8

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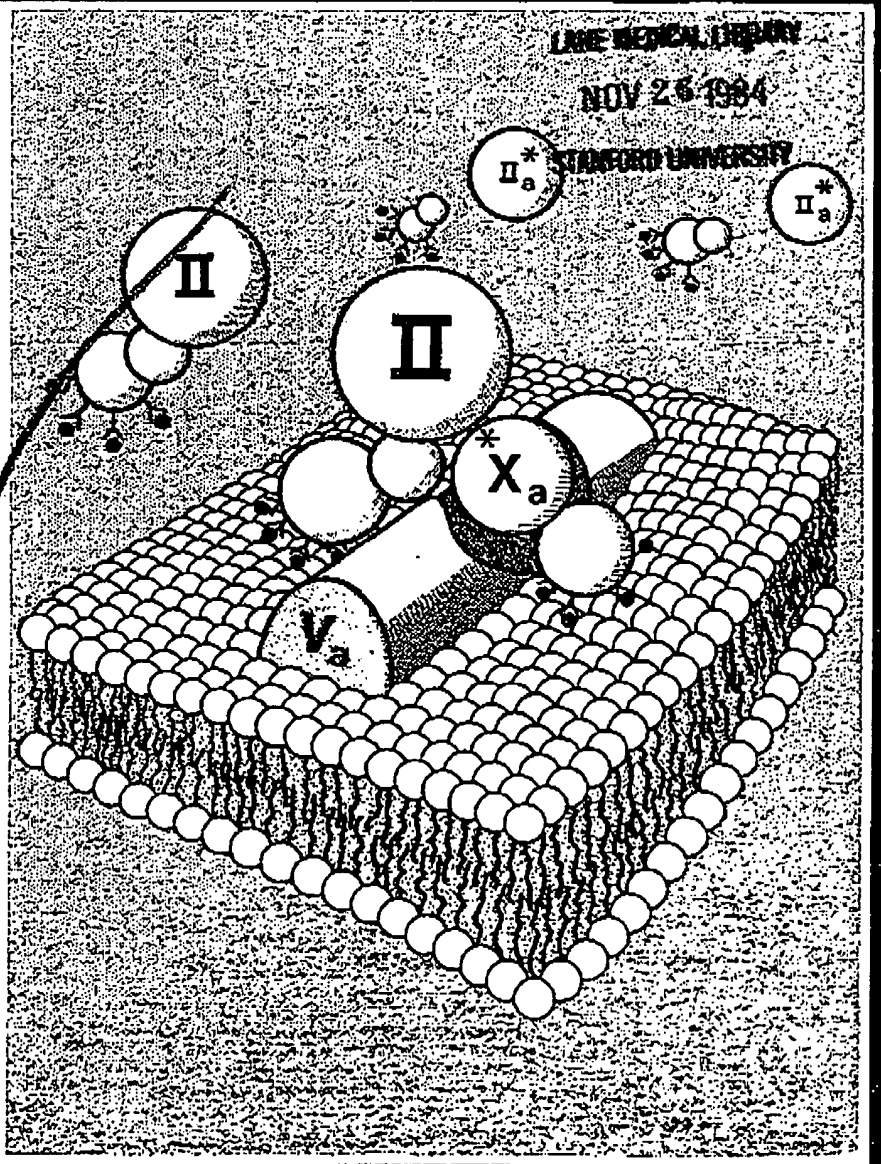

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The Effects of High Doses of Aspirin and Related Benzoic Acid Derivatives on Arterial Thrombosis in Male Rats[1]

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Abstract. The antithrombotic effects of four compounds structurally related to aspirin (acetylsalicylic acid, ASA) were examined in a rat model of arterial thrombosis and compared to ASA. ASA had antithrombotic activity, but only at high doses (200 mg/kg), when carotid artery thrombosis was induced 15 min after intravenous drug administration. Lower doses were associated with augmented thrombus formation in some animals. 2-Propionyloxybenzoic acid, which has in vitro activities similar to ASA, caused similar in vivo effects, but was antithrombotic at 100 mg/kg. 3-Propionyloxybenzoic acid, which augments platelet function in vitro, and 3-methylphthalide, which inhibits biphasic adenosine diphosphate-induced platelet aggregation, had no statistically significant effects. 2-Acetoxybenzoic acid, which is a weak platelet aggregation and prostaglandin biosynthesis inhibitor, had antithrombotic activity at 100 and 200 mg/kg and was not associated with augmented thrombosis at lower doses as found with ASA. The pattern of antithrombotic activity of this series of compounds does not reflect in vitro effects on prostaglandin biosynthesis and indicates alternative mechanisms of antithrombotic activity.

Introduction

Attempts to inhibit thrombus formation with aspirin (acetylsalicylic acid, ASA) in experimental models [1] and in clinical studies [2–5] have been less than completely successful; however, it has been found that ASA has a clearly beneficial effect in some groups of patients under some circumstances. The discovery of ASA-sensitive prostacyclin (PGI_2) biosynthesis [6] has provided a possible explanation for the variable in vivo results with ASA. One strategy for the development of antithrombotic therapy has been to maximize the platelet-inhibitory effects of ASA and minimize the inhibition of PGI_2 biosynthesis by using doses within a narrow range [7, 8]. In other studies, high doses of ASA have been found to inhibit platelet functions

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Table I. Summary of ASA-like compounds

Compounds	Platelet					Blood vessel		Rat paw edema [13]
	aggregation [13, 22, 23]		ATP release [23]	PG synthesis [13, 23]	PDE activity[1]	PGI_2 activity [23]	arachidonic acid metabolism [23]	
	1st phase	2nd phase						
ASA	–	++++	++++	++++	–	+++	++++	++
2-PBA	–	+++	+++	+++	–	+++	+++	+++
3-PBA	–P	–	P	–	–	P	–	P
ABA	–+	–+	–	–	–	–+	–+	++
3-MP	+++	+++	+++	–+	+++	+++	–	++++

+ = Inhibition; – = no effect; P = potentiation.
[1] Platelet phosphodiesterase activity [*Killackey* et al., unpublished].

and thrombus formation by mechanisms independent of cyclooxygenase inhibition [9–11].

This laboratory has been involved in the study of a series of ASA-like compounds which display a variety of effects in vitro on platelet function, platelet prostaglandin (PG) biosynthesis and vascular PGI_2 production, and in vivo on carrageenin-induced rat paw inflammation (table I), despite having only slight structural modifications compared to ASA. We now report on the effects of high doses of these compounds in a rat model of arterial thrombosis in which ASA has previously been shown to have antithrombotic effects [10, 11].

Methods

Experimental Agents

The compounds studied are shown in figure 1. ASA was obtained from Sigma (St. Louis) and 2-acetyloxybenzoic acid (ABA) from Aldrich (Montreal). The other compounds were synthesized in this laboratory

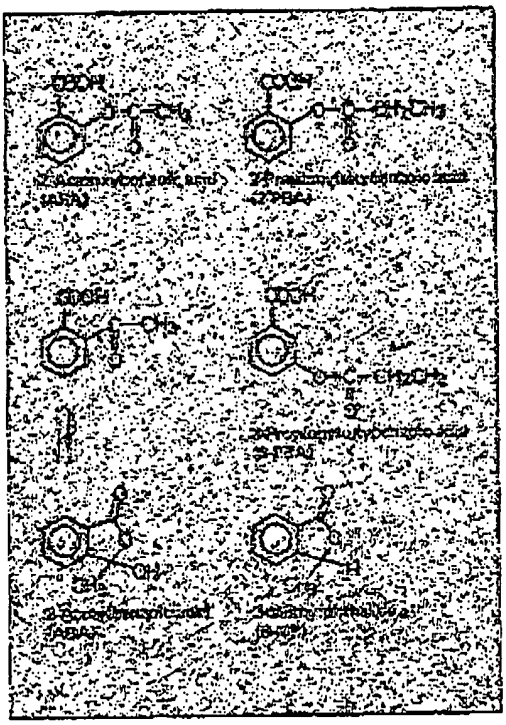

Fig. 1. Structure of ASA and related compounds.

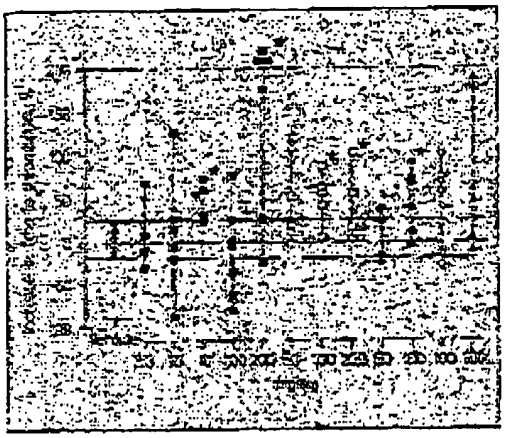

Fig. 2. The effects of ASA (■) and related compounds (□ = ABA, ● = 2-PBA, ○ = 3-PBA, ▲ = 3-MP) on the rat thrombosis model. Each point represents the difference in time to thrombosis of the left carotid artery (after drug administration) compared to the right carotid artery (control, x) as a percent of the control for a single animal. Doses are indicated on the abscissa. The range of values for control animals is also indicated. ASA 10' indicates that thrombosis was induced 10 min after 10 mg/kg ASA administration instead of the usual 15 min. Asterisks indicate statistically significant differences: ASA 10 mg/kg, $p < 0.005$; ASA 200 mg/kg, $p < 0.05$; ABA 100 mg/kg, $p < 0.01$; ABA 200 mg/kg, $p < 0.05$; 2-PBA 100 mg/kg, $p < 0.02$.

in collaboration with Dr. *M. Hirst* of this department. Details of the synthetic methods are available upon request. Urethane (ethyl carbamate) was from BDH (Toronto) and polyethylene glycol 600 was from Fisher (Toronto).

Thrombosis Model

Male Sprague-Dawley rats, 200–250 g in weight, were anesthetized with urethane (6 ml/kg of a 25% solution, i.p.). A tracheal cannula was inserted and the right and left common carotid arteries were dissected free of surrounding structures. Thrombosis was induced in the carotid artery by application of an electric current (350 V, 1 mA DC) for 1 min, based on the method of *Hladovec* [12]. In this model, downstream arterial temperature, as recorded with a thermistor probe, falls in proportion to the extent of arterial occlusion [10, 11]. Electron microscopic studies of thrombus formation in this model have demonstrated the presence of occluding thrombi, consisting of densely packed platelets, fibrin strands and red blood cells, at the time of maximum fall in temperature, while at earlier times, areas of platelet deposition only are found [10, 11]. The temperature was recorded for 15 min after termination of the electrical stimulus. Animal body temperature was maintained at 37 °C using a warmed surgical stage. After injury to the right carotid artery (control), vehicle (50% polyethylene glycol 600 in isotonic saline) or drug (dissolved in vehicle) was injected into the femoral vein of the right leg in volumes less than 0.6 ml. Thrombosis was induced in the left carotid artery 15 min after drug administration except in one group of rats where thrombosis was induced 10 min following ASA (10' mg/kg). The time (min) from the initiation of the electrical stimulus to the point at which the downstream temperature fell to 50% of the final value was defined as the time to half maximum fall in temperature ($T_{1/2}$max). The results were expressed as the increase in the $T_{1/2}$max of the test vessel compared to the control, as a percent of the control value. Drug effects on thrombus formation were determined by comparing the $T_{1/2}$max of the left artery (test) to the $T_{1/2}$max of the right artery (control) by Student's t test for paired data.

Results

The differences in the time to thrombus formation between the right carotid artery control and the left carotid artery after vehicle infusion were small (fig. 2). ASA caused an increase in the range of these values and, depending on the dose and time of stimulus administration, was associated with either the inhibition or augmentation of thrombosis. 3.3 mg/kg of ASA (i.v.) was the lowest dose studied as it inhibits rat platelet aggregation induced by arachidonic acid without in-

hibiting rat aorta PGI_2 production [*Killackey et al., unpublished*]. This dose of ASA had no consistent effect on thrombus formation. 10 or 100 mg/kg of ASA, administered 15 min before induction of thrombus formation, also had no consistent effect; however, with the higher dose, augmentation of thrombus formation occurred in 3 out of 7 animals, while thrombosis was inhibited in only 1 animal. ASA had statistically significant antithrombotic effects at high doses (200 mg/kg) and in 3 out of 6 animals vessel occlusion was completely inhibited. In the case where thrombosis was induced 10 min after administration of ASA (10 mg/kg), there was a statistically significant inhibition of thrombosis; however, this effect was transient. These findings are consistent with previously reported observations [10, 11].

Thrombosis was induced 15 min after the administration of all the other compounds. In previous studies, ABA has had no effects in vitro on platelet aggregation or ATP release or on platelet or blood vessel cyclooxygenase activity when tested at concentrations less than 1 m*M* (table I). ABA, however, is at least as effective as ASA in inhibiting carrageenin-induced rat paw edema [13]. Here, ABA caused trends toward the inhibition of thrombus formation and the effects were statistically significant at 100 and 200 mg/kg. In contrast to ASA, ABA had more consistent antithrombotic effects at 100 mg/kg than at higher doses and it was not thrombogenic at any dose. ABA differs chemically from ASA in that an acetyl group replaces the important acetoxy group of ASA. This allows ABA to exist in two tautomeric forms (fig. 1). 3-Methylphthalide (3-MP) was synthesized in order to have a compound which was similar to the ring tautomer form of ABA. Like ABA, 3-MP has no effect on platelet cyclooxygenase activity, but it does inhibit both phases of ADP-induced platelet aggregation (table I). 3-MP also inhibits PGI_2-like activity production from rabbit aorta rings, but does so without inhibiting rabbit aorta conversion of ^{14}C-arachidonic acid to PGs. Preliminary studies show that 3-MP inhibits cyclic nucleotide phosphodiesterase activity. Like ABA, 3-MP is also potent in vivo in inhibiting edema [13]. Here, 3-MP had antithrombotic activity in some animals but, unlike with ABA, the effects were not statistically significant. In addition, no augmentation of thrombosis occurred.

2-Propionyloxybenzoic acid (2-PBA) is structurally similar to ASA except that the acetoxy side chain is extended one carbon unit to a propionyloxy group (fig. 1). Like ASA, 2-PBA inhibits platelet aggregation and PG synthesis and blood vessel PGI_2-like activity production, but does so only at higher concentrations than ASA (table I). In vivo, however, 2-PBA was more effective than ASA in inhibiting edema [13]. In the present study, 2-PBA caused statistically significant inhibition of thrombus formation at 100 mg/kg in contrast to ASA, which was thrombogenic at this dose.

3-Propionyloxybenzoic acid (3-PBA) differs structurally from 2-PBA only in the position of the propionyloxy side chain, but this leads to major alterations in pharmacological properties. 3-PBA has been associated with increased platelet aggregation, ATP release and blood vessel PGI_2-like activity production (table I) and with increased edema in a carrageenin-induced rat paw model of inflammation. In this study, 3-PBA had no statistically significant effects on thrombus formation, although the time to thrombus formation was prolonged in 4 out of 7 animals.

Discussion

ASA administered 15 min before injury had a greater tendency to promote thrombus formation than any of the other agents. The thrombogenic effect of ASA was first seen at 3.3 mg/kg and became more pronounced at 10 and 100 mg/kg. This has also been reported in studies with rabbits [14, 15]. The antithrombotic effects of low and high doses of ASA in rats, as seen here, have also been previously reported [10, 11]. The transient nature of the low dose (10 mg/kg) effect is of interest; however, it limits the potential usefulness of this ASA dose in preventing thrombosis. With 200 mg/kg ASA, the balance of effect is clearly on the side of antithrombotic activity. ASA was the only agent to completely eliminate thrombus formation in any animals and this effect is very reproducible as indicated in other reports [10, 11].

The results with ABA are of particular interest as this compound is very similar in structure to ASA, with an acetyl group replacing the reactive acetoxy group of ASA. ABA does not acetylate the cyclooxygenase enzyme and has few in vitro properties in common with ASA; however, it does inhibit rat paw edema in vivo. Here, ABA did not have the thrombogenic effects of ASA when tested at 50–200 mg/kg (i.v.), suggesting that this property may be related to the inhibition of PG biosynthesis. Although ABA did not cause complete inhibition of thrombosis as seen with ASA at 200 mg/kg (i.v.), it caused the most consistent antithrombotic effects of all agents tested. These effects were more consistent at 100 than 200 mg/kg, indicating that they are not a result of nonspecific toxicity. 3-MP was synthesized to resemble the ring tautomer form of ABA and it is more active than ABA in all in vitro assays including inhibition of platelet function and PGI_2-like activity production (table I) and in vivo in the rat paw edema model [13]. The results with 3-MP here are variable compared to ABA, possibly underlining the importance of 3-MP-mediated inhibition of PGI_2 biosynthesis.

2-PBA caused statistically significant inhibition of thrombosis at 100 mg/kg but not at 50 mg/kg and it did not display the wider range of effects seen with ASA. The similarity of effects of 3-PBA (100 mg/kg), 2-PBA and ABA, despite vastly different in vitro profiles (table I), provides further evidence that these compounds at these high concentrations are exerting effects which are not related to the in vitro effects on platelet aggregation and platelet PG biosynthesis.

ASA has unique effects on thrombosis when tested in this model and compared to these structurally related agents. The thrombogenic effect of ASA, even at low doses, is worrisome in the consideration of a drug for use as an antithrombotic agent. The consistent antithrombotic effects of ABA, an agent with no cyclooxygenase inhibitory activity, are more encouraging and close comparisons of the mechanisms of action of high doses of ASA, ABA and 2-PBA in vitro on cyclooxygenase-independent systems are now warranted. Reports that ASA has biphasic inhibitory effect on platelets in vivo [16], that ASA inhibits platelet function independent of cyclooxygenase activity [9] and that metabolites of the lipoxygenase pathway may be involved in the effects of ASA [17–19] are potentially important. Recent studies have confirmed that high doses of ASA prolong the bleeding time in rats with a dose-response pattern that is different from salicylate [20]. This is of interest in light of the apparent aug-

mentation of the antithrombotic effect of low-dose ASA, 10 min before injury, by salicylate [21].

This study shows that high doses of these benzoic acid derivatives share some of the antithrombotic properties of high doses of ASA despite their variable effects on PG biosynthesis. This series of compounds is a useful tool for investigating the lipoxygenase pathway of platelets and endothelial cells and other potentially important mechanisms of antithrombotic action of high-dose ASA.

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